Histone Deacetylase Inhibition Modulates Adaptive and Innate Immunity

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Presented in Partial Fulfillment of the Requirements for graduation

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by

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Table of Contents

Table of Figures

Abstract

Epigenetic gene silencing has been associated with the onset and progression of a wide range of diseases, including cancer. Histone deacetylase enzymes (HDAC) influence the epigenome by covalently modifying histone tails promoting tighter nucleosomal structure and transcriptional silencing. Consequently, drugs that inhibit HDAC enzymes hold significant promise as specific and effective therapeutic agents. While HDAC inhibitors are presently being evaluated in clinical trials, little is known regarding the effects of HDAC inhibition on host immunity. Here we report that the broad spectrum HDAC inhibitor AR-42 (Arno Pharmaceuticals) exerts a dose-dependent inhibitory effect on innate and adaptive immune function. The observed inhibition of NK and T cell cytotoxicity occurred at concentrations (100 – 500nM) below the range found to elicit anti tumor activity against Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCL). Overnight incubation of purified NK cells in low doses of AR-42 also inhibited antibody dependent cellular cytotoxicity (ADCC) against rituximab-coated EBV+ LCLs. Overnight incubation of effector and LCL target cells in low doses of AR-42 (100 – 500nM) modulated expression of key regulatory proteins involved with cellular and adaptive immune responses (NKG2D, KIR, NKp46, NKp30, MHC I). AR-42 led to decreased granzyme B (GzB) and interferon-gamma (IFNy) release by activated NK and T cells. Similar effects with IFNy and GzB production were seen with the broad spectrum HDAC inhibitors SAHA and valproate, however, NK cell cytotoxic potential remained unchanged with these two HDAC inhibitors. Washout of AR-42 from cell cultures led to significant recovery of cytotoxic activity and IFNy release from both NK and T cell effectors suggesting that the inhibitory effect is transient, fully reversible and comparable to vehicle (DMSO) treated effector cells. The differential effects observed with

SAHA and VPA on cytotoxicity and cytokine release suggests individual HDAC inhibitor drugs may exhibit distinct immune-modulatory profiles. The data suggest broad spectrum class I and II HDAC inhibitors suppress the adaptive and innate immune responses and highlight the importance of performing immune laboratory correlative studies as these drugs are evaluated in clinical trials.

Introduction

Immune deficiency is an ever increasing problem, made evident in recent years by the global HIV/AIDS epidemic, the fact that organ transplantation is now at an all time high and a steadily approaching population explosion among geriatric patients. A myriad of complications accompany immune deficiency and it is linked to an increased risk of developing cancer, especially virally-associated malignancies. Examples of these types of disorders include human papillomavirus (HPV)-associated cervical and skin cancer, hepatitis B and C-associated hepatocellular carcinoma, and human T-lymphotropic virus (HTLV)-associated adult T cell leukemia. [1]

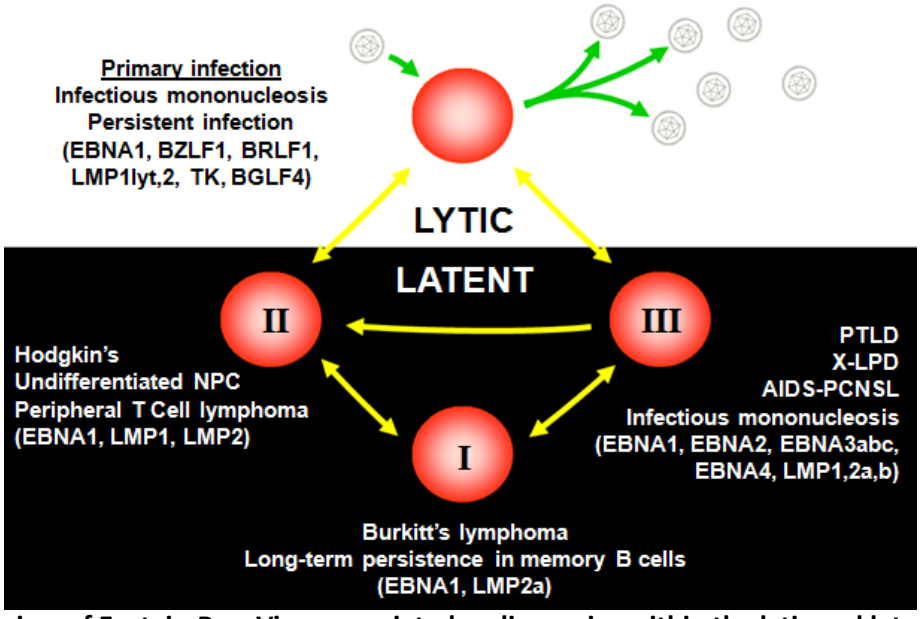


Figure 1. Overview of Epstein-Barr Virus-associated malignancies within the lytic and latent replication cycles.
The lytic and latent cycles of EBV-infected B cells is characterized by viral-protein expression including Epstein-Barr nuclear antigen (EBNA) and latent membrane protein (LMP). Lymphoblastoid cell lines, derived from healthy B cells and immortalized in culture, express a type III latency program.

The focus of our study is Epstein-Barr virus (EBV), which is a ubiquitous human herpes virus that infects B lymphocytes and epithelial cells in over 90% of the adult population. [1] EBV is

interesting because it can directly induce B lymphocyte transformation and is associated with a variety of neoplastic diseases that can each be identified by characteristic patterns of latent viral gene expression including Hodgkin's lymphoma, Burkitt's lymphoma, and post-transplant lymphoproliferative disorder (Figure 1). [2] High mortality rates associated with most EBV-associated malignancies underscore the need for effective treatment strategies. [3, 4] However, traditional chemotherapy and radiation therapy are unsuitable for this purpose as non-discriminate tumor-killing agents can cause further immunological compromise.

Within recent decades, newly developed chemotherapeutics work to target tumor-specific mechanisms with the goal of increasing efficacy and decreasing negative side effects. Current targeted treatment strategies for EBV-associated lymphoproliferative disorders include: anti-CD20 monoclonal antibodies; generating EBV-specific cytotoxic T lymphocytes (CTL) in laboratory cultures; or reducing the dosage of immunosuppressant drugs when applicable. [5, 6] While these treatment methods provide a somewhat acceptable level of treatment given the aggressiveness of EBV-associated lymphomas, there still exists significant room for improvement. [6]

Recent studies have indicated that EBV infection triggers epigenetic events that enable the progression of healthy B cells into cancerous B cells (Figure 2A). [7, 8] Modulation of chromatin remodeling mechanisms has been shown to be useful anti-tumor agents in a range of disorders and may hold promise for treating EBV^+ malignancies (Figure 2B). [9]

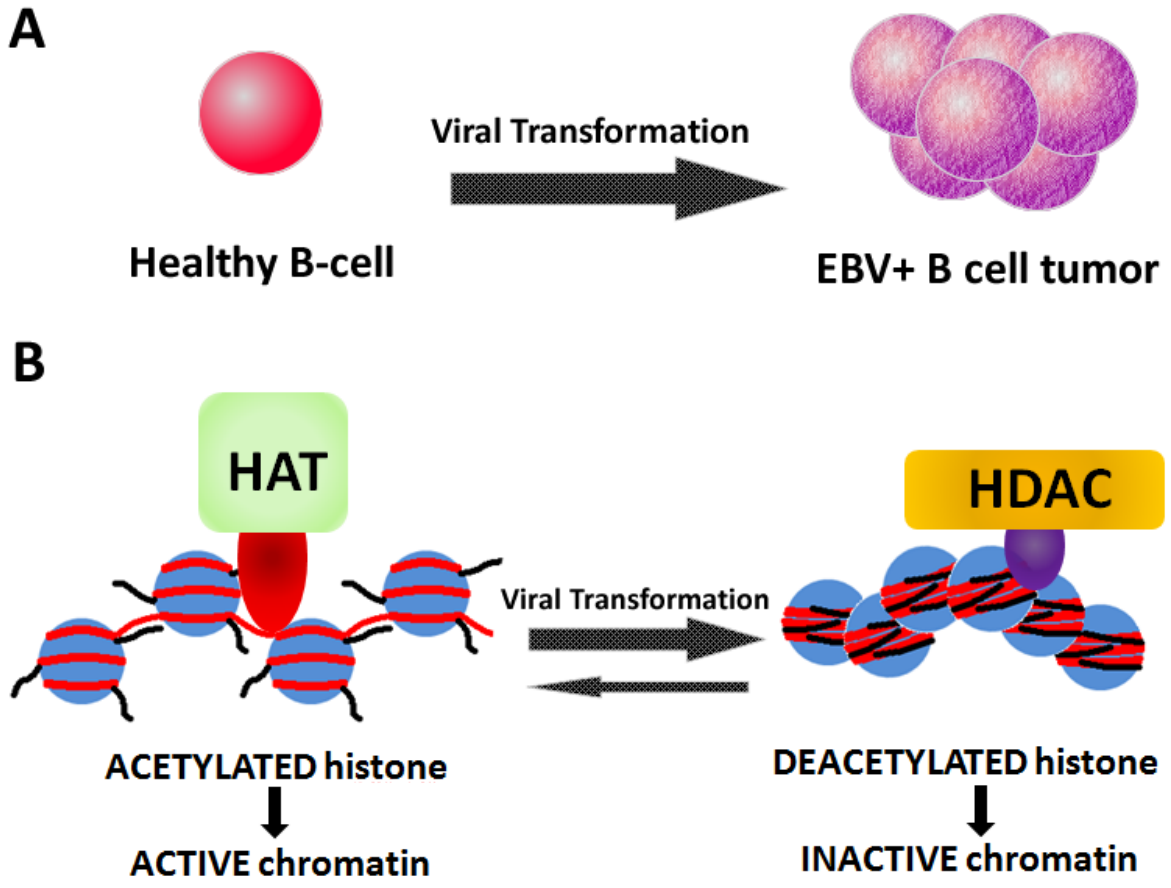


Figure 2. The HAT and HDAC functional equilibrium is disturbed in B cells during EBV–induced transformation to produce a more constricted genome.
(A) Healthy primary B-cells exposed to EBV in culture leads to EBV-infected B cell transformation which continually divide and exist as a tumor. (B) Current data suggests EBV-transformed cells utilize shift the HAT-HDAC equilibrium to deactivate key portions of the chromatin and decrease expression of tumor-suppressing genes. Inhibiting HDAC enzymes may promote a more relaxed nucleosomal state which could cause re-expression of tumor-suppressing genes and eventually EBV-LCL tumor death.

The epigenetic mechanism we investigated involved regulation of the nucleosome, which consists of DNA wrapped around a histone protein. Removal of acetyl groups from histone tails by histone deacetylase (HDAC) results in positively charged tails due to exposed lysine residues on its terminal amino acid chain. The positively charged histones bind electrostatically to negatively charged phosphate groups found in DNA. [9] Such a constricted nucleosomal state

prevents transcription factors from accessing and expressing genes, without base-pair modification of the genome. [10] Conversely, acetylation of the histone lysine tail by histone acetyltransferase (HAT) neutralizes the histone tail, which subsequently permits DNA dissociation and a relaxed nucleosomal state. [9] This relaxed nucleosomal state permits transcription factor-mediated gene expression of important regulatory genes required to maintain cellular homeostasis.

Previous research has shown HDAC activity is upregulated in numerous solid tumor malignancies. [11] As such, HDAC inhibitors have been developed to utilize this potential therapeutic target and have shown promise against behavioral, solid tumor, and hematological malignancies. [9, 10, 11] HDAC-I are becoming more prominent in clinical trials and have shown great potential in treating cancer, but relatively little is known of HDAC-I's effect on human immunity. [9]

The current model of the human immune system incorporates a vast array of cells, proteins, and metabolites working synergistically and antagonistically to create a redundant, buffered network of signaling, recognition, and removal of foreign material from a host; the immune system is complex with a fair degree of redundancy. The immune system consists of two main branches: innate immunity and adaptive immunity. Briefly, the innate immune response works to inhibit, kill, and/or remove non-host antigenic material via relatively non-specific means: a natural killer (NK) cell can destroy a broad array foreign organisms immediately without prior exposure to the foreign organism (Figure 3A). [13] Conversely, the adaptive immune response

fulfills similar immune-system roles via a more target-specific mechanism: a naive T cell requires auxiliary cells in order to activate and become an antigen-specific T cell capable of killing a specific foreign organism (Figure 3B,C). [13]

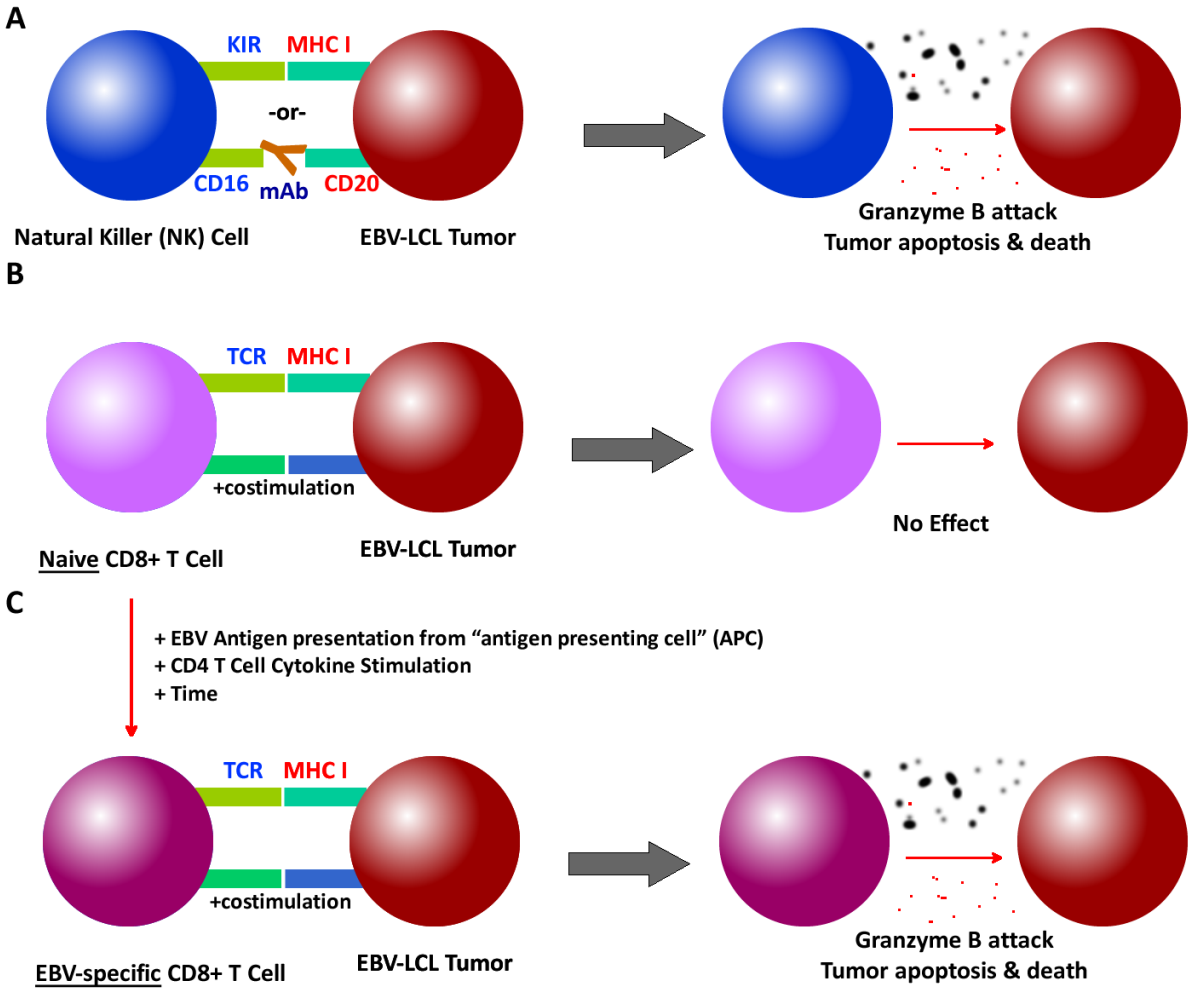


Figure 3. Overview of NK (innate) and T cell (adaptive) immune response against EBV-LCL tumor cells. (A) NK cells can recognize EBV-LCL tumors through recognition of antigen on class I major histocompatibility complex (MHC I) via KIR receptors, or through the use of soluble monoclonal antibodies specific for the EBV-LCL tumor via an Fc receptor (CD16). Proper NK recognition and signaling will lead to a granzyme B-mediated cytotoxic response resulting in programmed cell death (apoptosis) of the EBV-LCL tumor. (B) Naïve CD8+ cytotoxic T lymphocytes (CTL) are unable to initiate an attack against EBV-LCL tumors as CTLs must first be activated in order to attack a specific target. (C) Antigen presenting cells within the immune system must first present EBV antigens and other stimulating factors to induce transformation from a naïve CTL to an activated EBV-specific CTL. Upon recognition of an EBV-LCL tumor by EBV-specific CTL via the T cell receptor (TCR), granzyme B-mediated cytotoxic response is initiated to destroy the EBV-LCL tumor.

While both NK and T cells have differing requirements for killing antigenic matter, they share similar mechanisms for initiating target cell death. [14] Specifically, both cell types utilize a granzyme B-associated cell-mediated cytotoxicity (killing) response, the primary mechanism by which NK and T cells destroy foreign organisms (Figure 3). [14]

Given that several HDAC-I are presently being used treat patients with lyphoproliferative disorders, [15] it is critical to understand the effects of this unique class of drugs on human immunity. Earlier experiments conducted suggest that HDAC-I are non-toxic to healthy lymphocytes. [16] We examined three Zn^{2+} short-chain fatty acid-based HDAC inhibitors on host immune functionality: SAHA (suberoylanilide hydroxamic acid; vorinostat), valproate (valproic acid; depakote), and AR-42 (Arno Therapeutics). We hypothesized that AR-42, currently in phase 1 clinical trials, is capable of targeting EBV+ transformed B cells (tumor) without disturbing tumor-specific innate and adaptive cellular immune surveillance and cytotoxic mechanisms.

Materials and Methods

Cell Lines and Reagents

EBV-transformed LCL lines were generated from isolated donor B cells infected with EBV B95-8. Peripheral blood mononuclear cells (PBMCs) for EBV antigen-specific cytotoxic T lymphocyte (CTL) generation were isolated from donor blood using Ficoll-Paque (GE Healthcare, Piscataway, NJ). NK cells or PBMCs were isolated from leukopacks purchased from the American Red Cross (Columbus, OH) using a negative isolation RosetteSep NK isolation cocktail from StemCell Technologies (Vancouver, BC, Canada). Cells were cultured in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA), 10% sterile heat-inactivated fetal bovine serum (Equitech-Bio, Kerrville, TX), and antibiotic-antimycotic (Gibco Invitrogen, Carlsbad, CA). Recombinant human IL-2 (Proleukin®; specific activity = $18x10^6$ IU/ml) was reconstituted in 0.1% bovine-serum albumin (BSA) and 0.9% saline and provided by Chiron Corporation (Emeryville, CA). Human recombinant IL-12 reconstituted in 0.1% BSA and phosphate-buffered saline (PBS) was provided by Genetics Institute (Cambridge, MA) and gifted from Dr. William Carson III (Columbus, OH). Phorbol myristate acetate (PMA) was obtained from MP Biomedicals (Solon, OH). Ionomycin (INO) was obtained from Sigma-Aldrich (St. Louis, MO). Radioactive Cr-51 was obtained from Perkin Elmer (Waltham, MA) and gifted from Dr. John C. Byrd (Columbus, OH). Enzyme-linked immunosorbent spot assay (ELISPOT) coating human interferon-γ (IFNγ) monocloncal antibody (mAb) (1-D1K), coating human granzyme B mAb (GB10), developing biotinylated human IFNγ mAb (7-B6-1), developing biotinylated human granzyme B mAb (GB11), Streptavidin-Alkaline Phosphatase, and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt)/NBT (Nitro-Blue Tetrazolium Chloride) was obtained from MABTECH (Cincinnati, OH). The ELISPOT

MAIPS4510 plate was obtained from Millipore (Burlington, MA). Annexin V FITC, annexin binding-buffer, propidium iodide (PI), mouse anti-human CD8 PE mAb, mouse anti-human MIC A/B PE mAb, mouse-antihuman class I PE mAb, mouse anti-human CD158b (KIR) PE mAb, mouse anti-human NKp46 PE mAb, mouse anti-human NKp30 PE mAb, and mouse anti-human NKG2D PE mAb was obtained from BD Pharmigen (San Jose, CA). Mouse anti-human CD69 PE mAb was obtained from eBiosciences (San Diego, CA). Normal mouse IgG was obtained from Sigma-Aldrich (St. Louis, MO). RAKFKQLL EBV Pentamer APC (190-B*0801) was obtained from ProImmune (Bradenton, FL). AR-42, previously known as OSU-HDAC42, was provided by Ching-Shih Chen (Columbus, OH). Suberoylanilide hydroxamic acid (SAHA) was gifted by Dr. John C. Byrd (Columbus, OH). Sodium valproate was obtained from Alexis Biochemicals (San Diego, CA) and gifted by Dr. Michael Grever (Columbus, OH). All incubations were conducted at 37°C, 5% CO_2 unless otherwise stated.

LCL Viability Assay

$1x10^6$ cells/ml of EBV-LCLs were cultured in 6-well plates, and cell viability was assessed using a FITC annexin-PI apoptosis detection kit (BD Pharmigen Biosciences, San Diego, CA). LCLs treated with different concentrations of HDAC inhibitors or DMSO control for 24, 48, 72, and 96 h. Cells ($5x10^5$) were harvested, washed with cold PBS, and resuspended in annexin binding-buffer containing annexin and PI in the dark for 15 min at room temperature. Cells were analyzed by flow cytometry for annexin and PI negative staining to determine cell viability.

Activating and expanding EBV-antigen specific cytotoxic T lymphocytes

Healthy donor PBMCs and irradiated autologous EBV-LCLs (Irradiator) were combined at a 1:1 ratio and incubated in 96-well round-bottom plates at $0.5x10^6$ cells/ml with 100 u/ml IL-2. Cells were expanded to 24-well plates with additional medium as necessary during a 12-14 day period. The presence of EBV-antigen specific CTLs was determined by mouse anti-human CD8 PE and EBV-tetramer RAK APC flow cytometry staining. The PBMCs containing activated EBV-specific CTLs were either incubated overnight in HDAC-I drugs, or further stimulated with irradiated EBV-LCLs and incubated for an additional 4-5 days for washout studies.

Radioactive chromium (^{51}Cr) release cytotoxicity assay

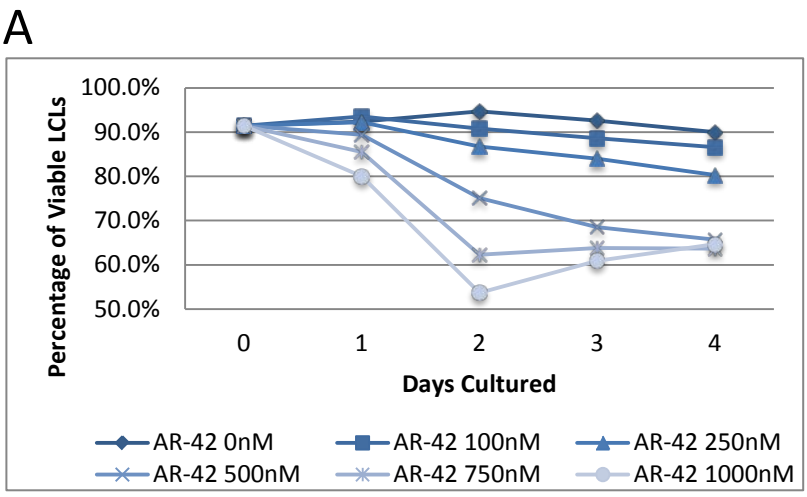
A standard 4 h chromium release assay was performed as previously described. [17] Briefly, healthy LCLs were labeled with ^{51}Cr at a concentration of 0.15 mCi $^{51}Cr/1x10^6$ cells for 1.5 h. Labeled LCLs were washed twice with RPMI 1640, incubated with 10ug/ml Rituximab or Herceptin as appropriate, and plated into 96-well V-bottom plates at $5x10^3$ cells/well. Effector cells (NK, T) were plated at 50:1, 25:1, 12.5:1, 6.25:1, or 0:1 effector:target ratios into the wells containing the labeled LCLs, and incubated for 4 h. Sodium dodecyl sulfate (SDS) was used to lyse LCL-only wells to determine the maximum gamma release count. Supernatant was harvested and radioactivity measured in a gamma counter. Relative cytotoxicity calculations were determined by: % lysis = 100 x (ER – SR)/(MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively. [17]

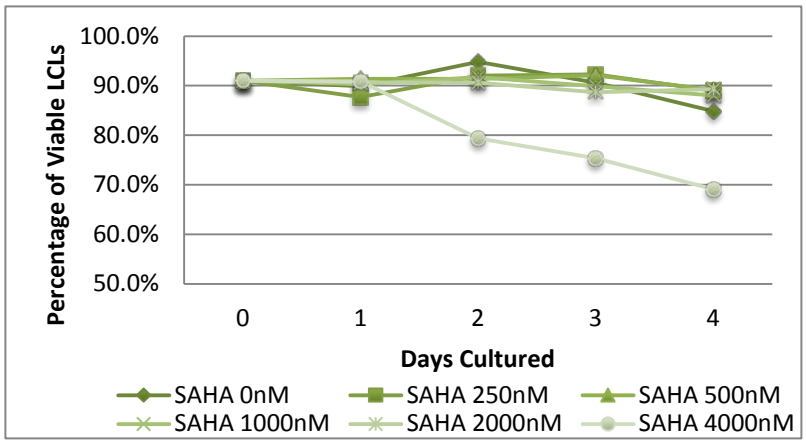
ELISPOT for IFNy and granzyme B release

A human IFNy and granzyme B kit from MABTECH AB was used to determine NK cell and T cell cytotoxicity. Briefly, a Millipore MAIPS plate was incubated with coating IFNy or granzyme B antibody overnight, washed out with phosphate buffered saline (PBS), and $5x10^4$ HDAC-treated effector cells added. Inflammatory cytokines IL-2 (100 u/ml) and IL-12 (10ng/ml) were used to stimulate NK cells, or IL-2 and EBV-LCLs (5:1 effector:target) to stimulate T cells. Plates were incubated for 18 h at 37°C 5% CO_2, washed with PBS, and incubated with detection IFNy or granzyme B antibody for 2 h at room temperature. Plates were washed with PBS, incubated with Streptavidin-ALP for 1 h at room temperature, and developed with BCIP/NBT substrate solution. Plates were dried overnight and analyzed on an ELISPOT reader.

Results

HDAC inhibitors affect EBV+ LCL growth in a dose dependent fashion





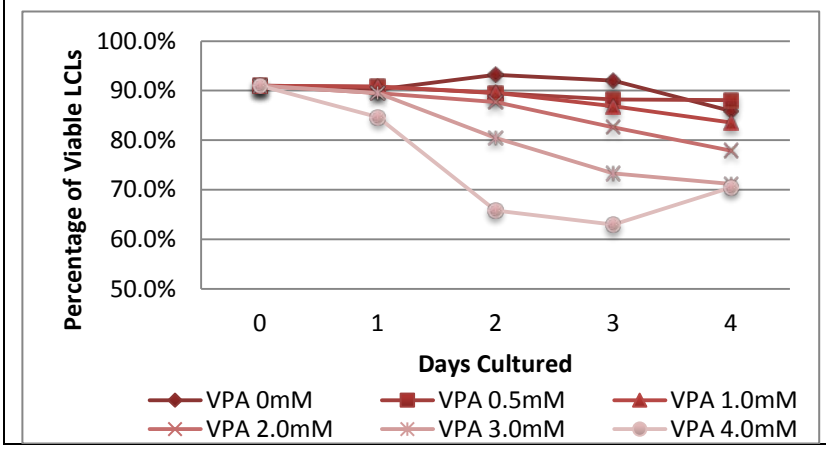


Figure 4. HDAC inhibitors affect EBV+ LCL viability in a dose dependent fashion.
(A) EBV+ LCLs generated from a B95.8 EBV strain cultured in AR-42. Viability was then assessed by annexin-PI flow cytometry every 24 hours. (B) SAHA (C) Valproate

An annexin-PI viability assay were used determine the effect of HDAC inhibition on EBV+ lymphoblastoid cell lines (LCL). LCLs were cultured in media that contained: 0, 100, 250, 500, 750, & 1000 nM AR-42; 0, 250, 500, 1000, 2000, 4000 nM SAHA; 0, 0.5, 1, 2, 3, 4 mM valproate for 4 days (Figure 4). B cell populations that were cultured in 250-1000 nM AR-42 concentrations experienced significant cell death by day 2 (87, 75, 62, & 54% viability, respectively) (Figure 4A). Conversely, the B cell population that was cultured in 750 and 1000 nM AR-42 actually displayed higher viability by day 4 than the day 2 population. This may be due to resistance mechanisms developed at doses of AR-42 at or above 750 nM. B cell populations cultured in 250-2000 nM SAHA resulted in little cell death during the 4 day incubation (Figure 4B). Significant B cell death was observed in the 4000 nM SAHA condition by day 4 (69% viability). Class I-specific HDAC-I valproate resulted in some B cell death by day 3 at 2-4 mM (83, 73, & 63% viability, respectively) (Figure 4C). By day 4, the 4 mM condition's viability recovered (71%) in a fashion similar to AR-42 at higher doses. Based on the LCL growth inhibition results, we used similar concentration ranges of each HDAC inhibitor in subsequent cellular immunity experiments.

AR-42 inhibits NK cell-driven ADCC against autologous EBV+ LCL targets

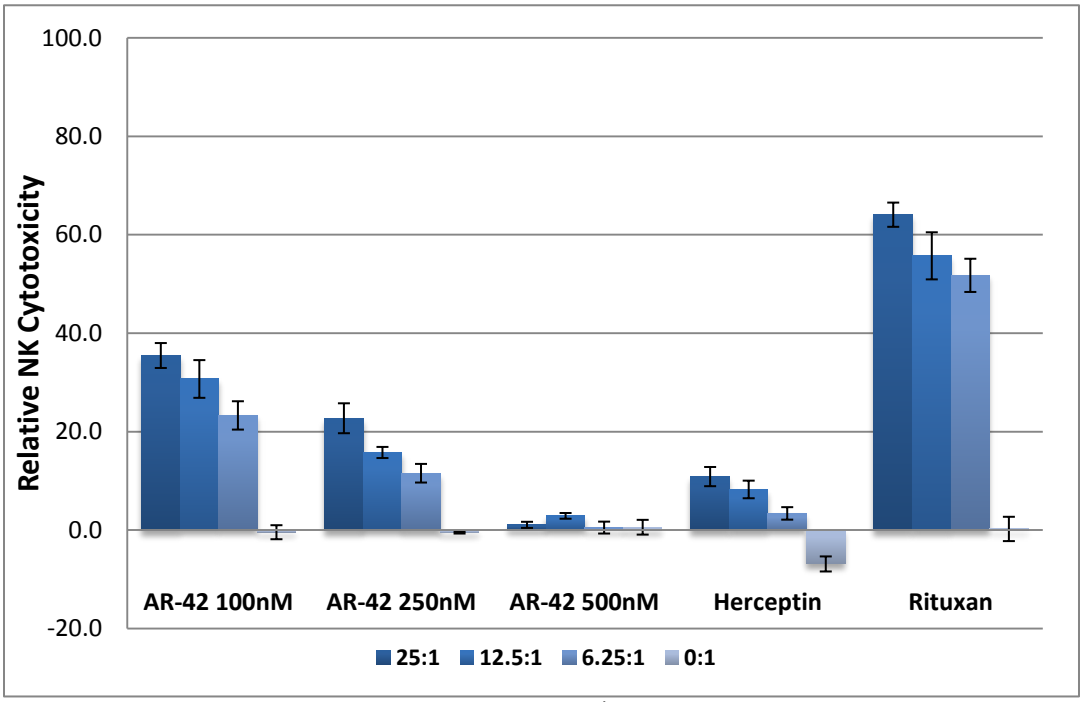


Figure 5. AR-42 inhibits NK cell-driven ADCC against EBV$^+$ LCLs.
NK cells purified from donor blood was cultured overnight in IL-2 and AR-42. Cytotoxicity was determined by a 51Cr release assay with effector-target ratios of 25, 12.5, 6.25, and 0 to 1. Rituximab (anti-CD20 mAb) was used in all AR-42 conditions, with Herceptin (anti-erbB2) as the negative antibody control.

We used a four hour 51Cr release assay to assess the effect of AR-42 on NK cell-driven antibody dependent cellular cytotoxicity (ADCC) (Figure 5). Rituximab (anti-CD20) was the antibody of choice for the assay because of its wide clinical use and proven efficacy against B cell lymphomas that express CD20 antigen. Herceptin (anti-erbB2), a commonly used breast cancer drug, was used as a negative control. NK cells that were not treated with AR-42 overnight displayed high relative ADCC at both 25:1, 12.5:1, & 6.25:1 effector-target (E:T) ratios in combination with rituximab (64, 56, & 52%, respectively). NK cells treated overnight with 100-250 nM AR-42 displayed a reduced capability to kill target B cells in combination with rituximab at the highest E:T ratio (36 and 23%, respectively). Treatment with 500 nM AR-42 limited NK

cytotoxicity significantly (1.1%). AR-42 exhibited a clear dose-dependent inhibitory effect on NK cytotoxicity against EBV LCLs at concentrations below the MIC (minimum inhibitory concentration) required for significant LCL growth inhibition.

AR-42 inhibits CTL direct cytotoxicity against autologous EBV+ LCL targets

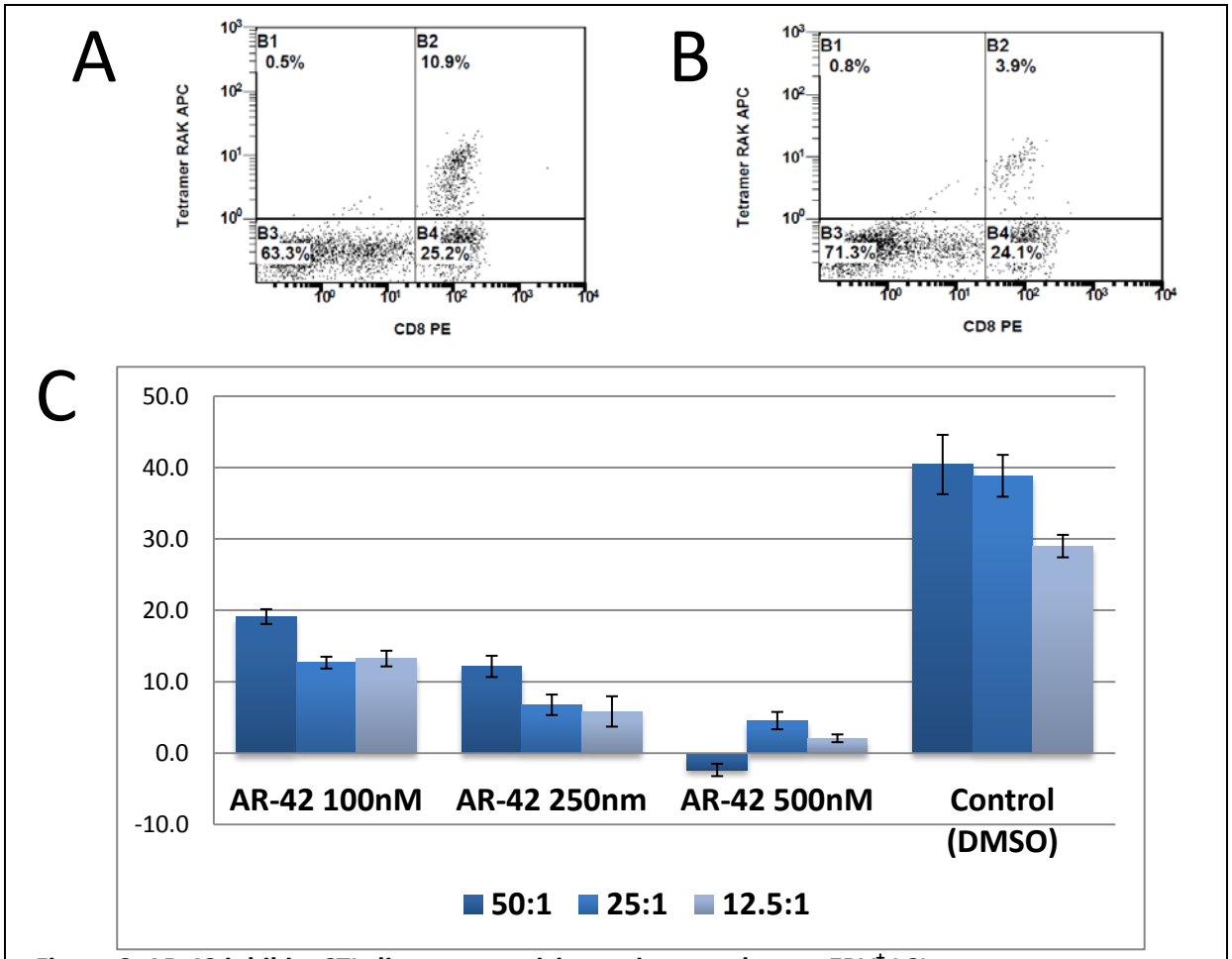


Figure 6. AR-42 inhibits CTL direct cytotoxicity against autologous EBV$^+$ LCLs.
(A) Healthy donor PBMCs activated and expanded against autologous LCLs for 12 days. with IL-2 and stained for EBV-specific CTLs (CD8$^+$/RAK$^+$). (B) Non-LCL stimulated PBMCs were also stained to determined baseline EBV-specific CTLs (CD8$^+$/RAK$^+$). (C) The PBMCs containing activated EBV-specific CTLs was incubated overnight in AR-42. Cytotoxicity against labeled LCLs was determined by a ^{51}Cr release assay with effector-target ratios of 50, 25, and 12.5 to 1.

A four hour 51Cr release assay was performed on activated EBV-specific CTLs against autologous EBV+ LCL targets and an effector-target ratio of 50:1, 25:1, and 12.5:1 (Figure 6). The CTLs treated overnight with 100 and 250 nM AR-42 indicated a reduced capability to kill target B cells at the highest E:T ratio (19 and 12%, respectively). Treatment with 500 nM AR-42 limited CTL cytotoxicity significantly (-2%). The observed dose-dependent cytotoxicity results in AR-42-treated CTLs resembled that of AR-42-treated NK cells.

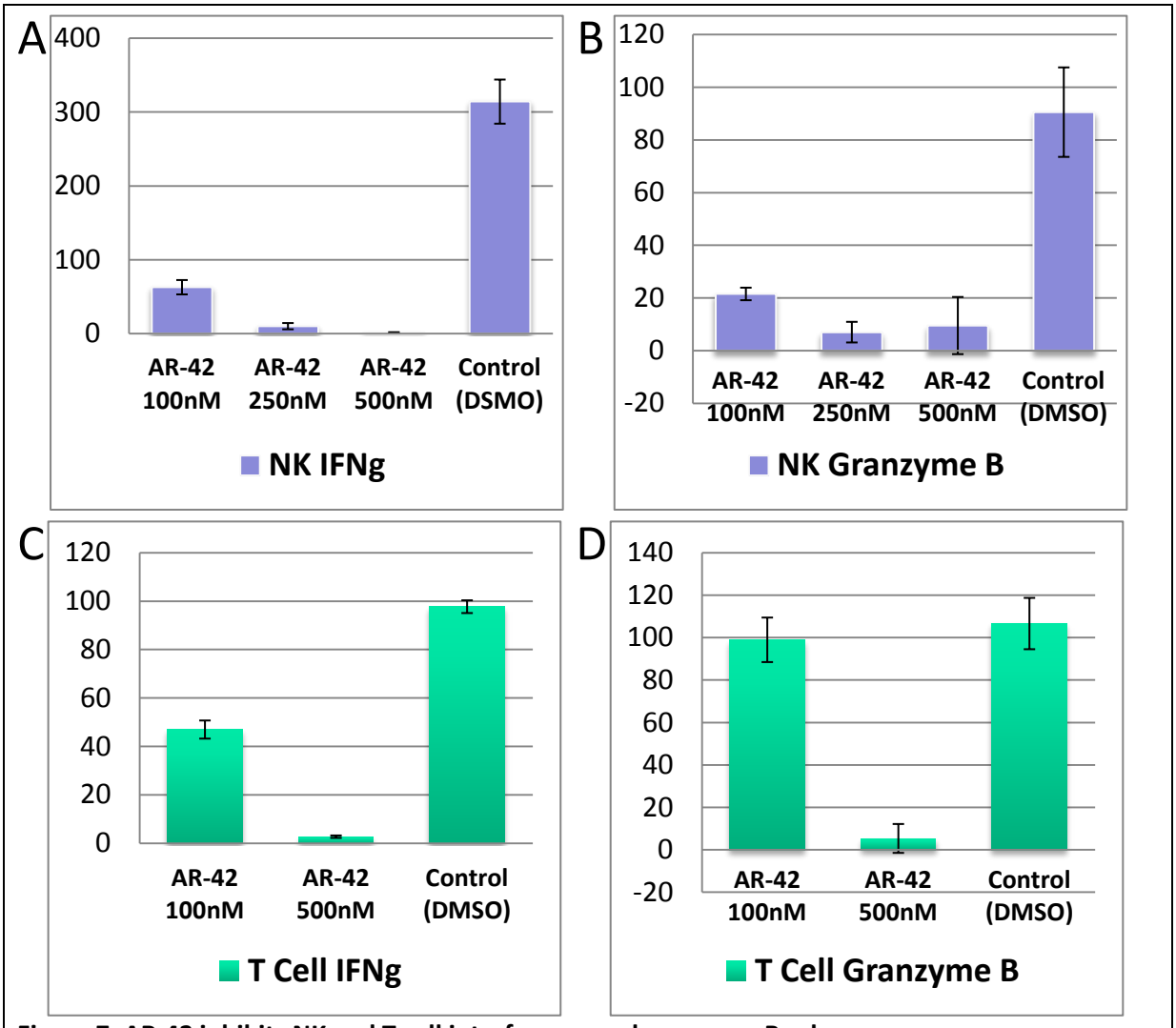


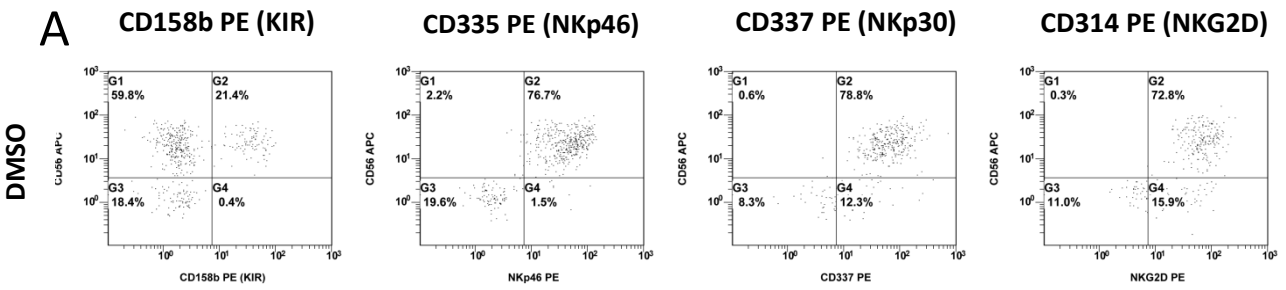
Figure 7. AR-42 inhibits NK and T cell interferon-γ and granzyme B release.

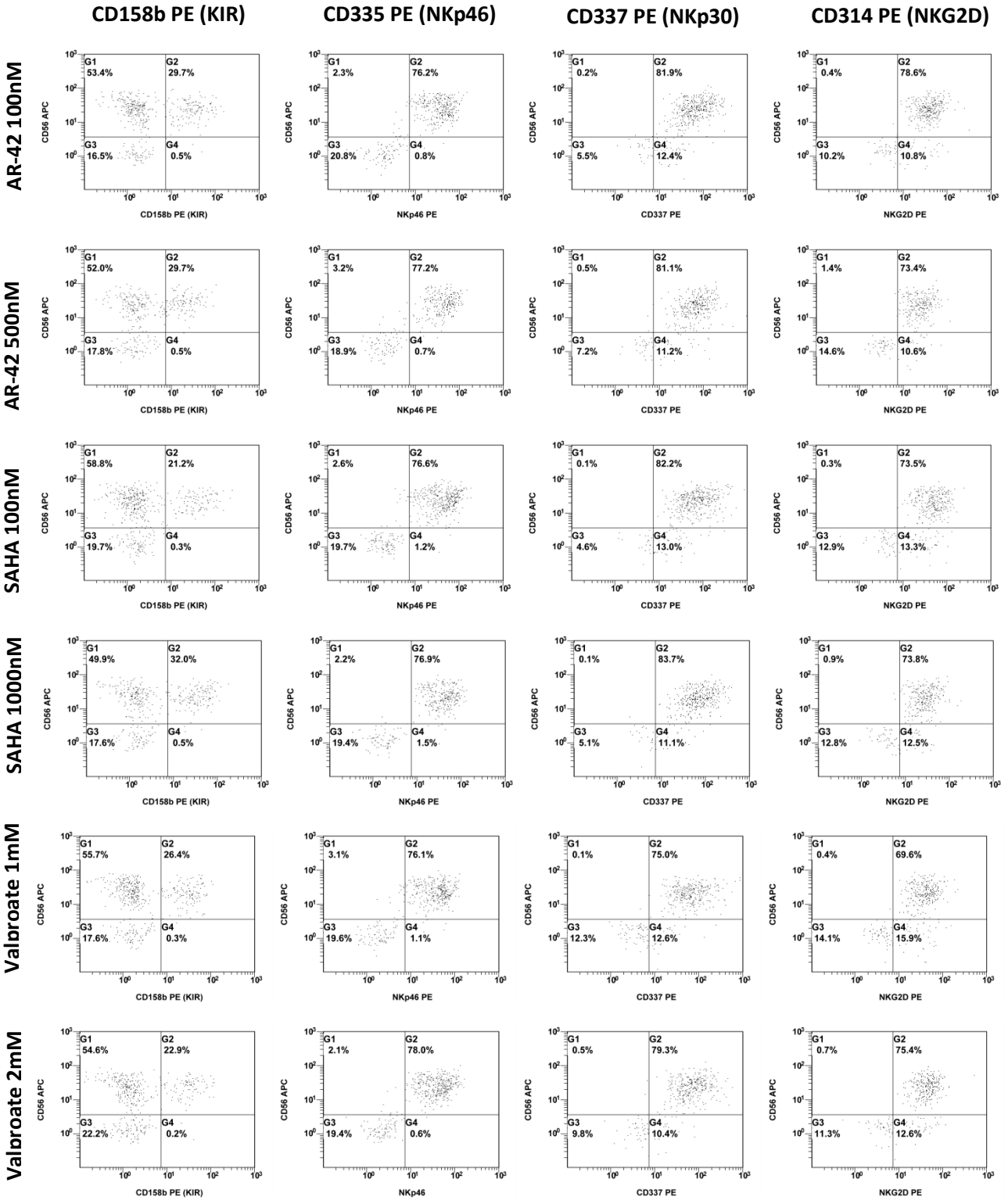
(A) Purified NK cells cultured in IL-2 and AR-42 overnight. Cells were further stimulated with IL-2 and IL-12 for 18 hours in an ELISPOT assay and checked for IFNγ or (B) granzyme B. (C) Healthy donor PBMCs activated and expanded against autologous LCLs for 12 days with IL-2 was incubated for 4 days in AR-42. Cells were stimulated with IL-2 and LCLs for 18 hours and checked for IFNγ or (D) granzyme B.To further examine AR-42's observed inhibitory effect on NK and CTL cytotoxicity, AR-42 treated effectors were tested for interferon-γ and granzyme B secretion potential.

AR-42 inhibits NK and T cell interferon-γ and granzyme B release

Purified NK cells or activated EBV-specific T cells were initially treated overnight (NK) or for 4 days (T) with 100-500 nM AR-42 and further stimulated with inflammatory cytokines (IL-2, IL-12) or EBV+ LCLs in an ELISPOT assay to determine IFNγ and GzB secretion potentials (Figure 7). NK cells treated with 100, 250, and 500 nM AR-42 produced significantly less IFNγ and GzB (63, 10, 1 spots and 22, 7, 10 spots, respectively) in a dose-dependent manner compared to the untreated control (314 and 91 spots, respectively). T cells treated with 100 and 500 nM AR-42 also resulted in decreased IFNγ and GzB secretion (47, 3 spots and 99, 5 spots, respectively) compared to the untreated control (98 and 107 spots, respectively). The 100nM nM AR-42 CTL condition did not significantly reduce GzB secretion levels compared to the control. These data suggest CTL cytotoxicity inhibition observed with HDAC inhibitors may not be granzyme B-mediated.

HDAC inhibitors modulate activating and inhibitory markers on NK cells




CD158b PE (KIR)
CD335 PE (NKp46)
CD337 PE (NKp30)
CD314 PE (NKG2D)
AR-42 100nM
AR-42 500nM
SAHA 100nM
SAHA 1000nM
Valproate 1mM
Valproate 2mM
CD56 APC
CD158b PE (KIR)
NKp46 PE
CD337 PE
NKG2D PE
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G4 12.6%

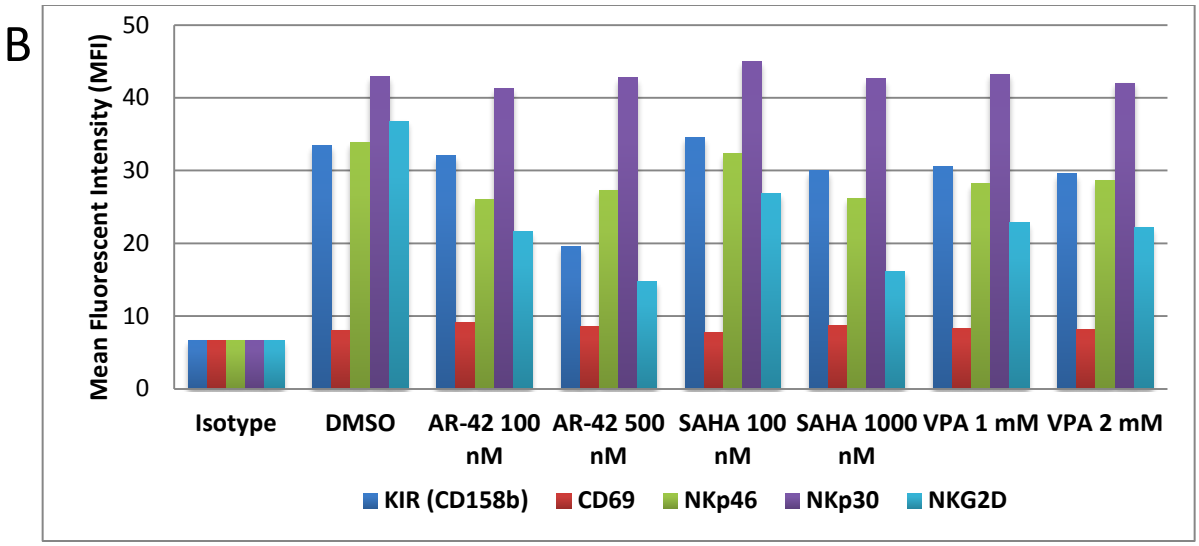


Figure 8. HDAC inhibitors modulate activating and inhibitory markers on NK cells.
(A) Purified NK cells treated overnight in IL-2 and AR-42, SAHA, or valproate. Markers were stained with fluorescent mAbs and analyzed via flow cytometry with gating for lymphocytes and $CD56^{+}$ events. CD69 not shown. (B) Chart indicating mean fluorescent intensity values.

We used flow cytometry to determine if AR-42's observed inhibitory effect on NK cytotoxicity was mediated by activation or inhibitory receptors (Figure 8A). Briefly, NK cells and EBV+ LCLs were incubated separately in AR-42, SAHA, or valproate (100 & 500 nM, 100 & 1000 nM, 1 & 2 mM, respectively) and control (DMSO) for 24 hours. Viable cells were then collected and stained with mAbs and analyzed via flow cytometry. AR-42 and SAHA both down modulates expression of the activating NK cell receptor NKG2D on purified CD56+ NK cells. AR-42 downmodulates the inhibitory receptor KIR on NK cells, especially at 500 nM. Mechanism not known. Little change in NK receptor expression was observed with HDAC-I valproate or NKp30 expression.

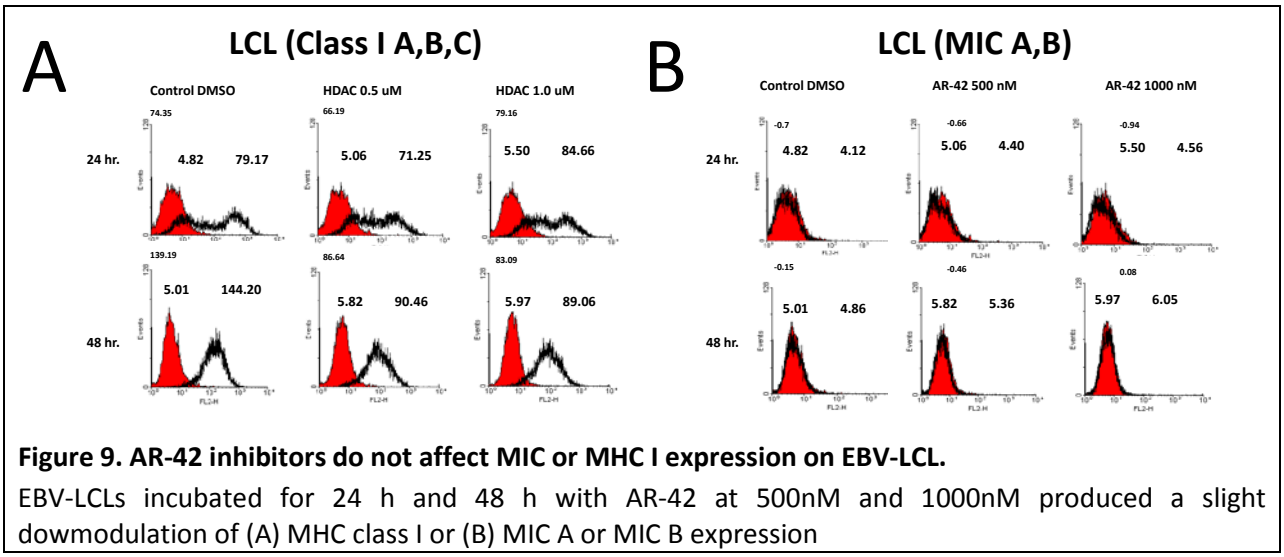
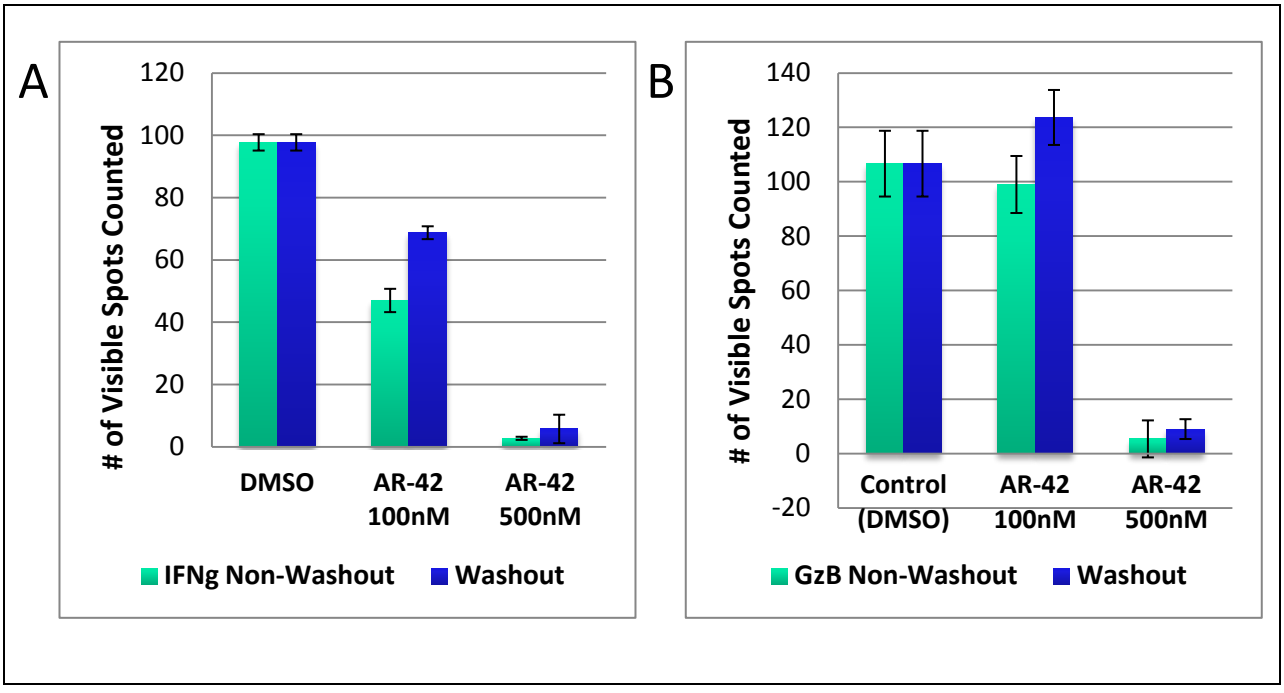


Figure 9. AR-42 inhibitors do not affect MIC or MHC I expression on EBV-LCL.
EBV-LCLs incubated for 24 h and 48 h with AR-42 at 500nM and 1000nM produced a slight dowmodulation of (A) MHC class I or (B) MIC A or MIC B expression

AR-42 also appeared to down modulate MHC Class I expression (KIR ligand) on EBV+ LCLs at higher concentrations (Figure 9A) while Mic A/B (NKG2D ligand) was unaffected (Figure 9B).

HDAC-I-mediated inhibition of antigen specific T cell responsiveness is transient and reversible



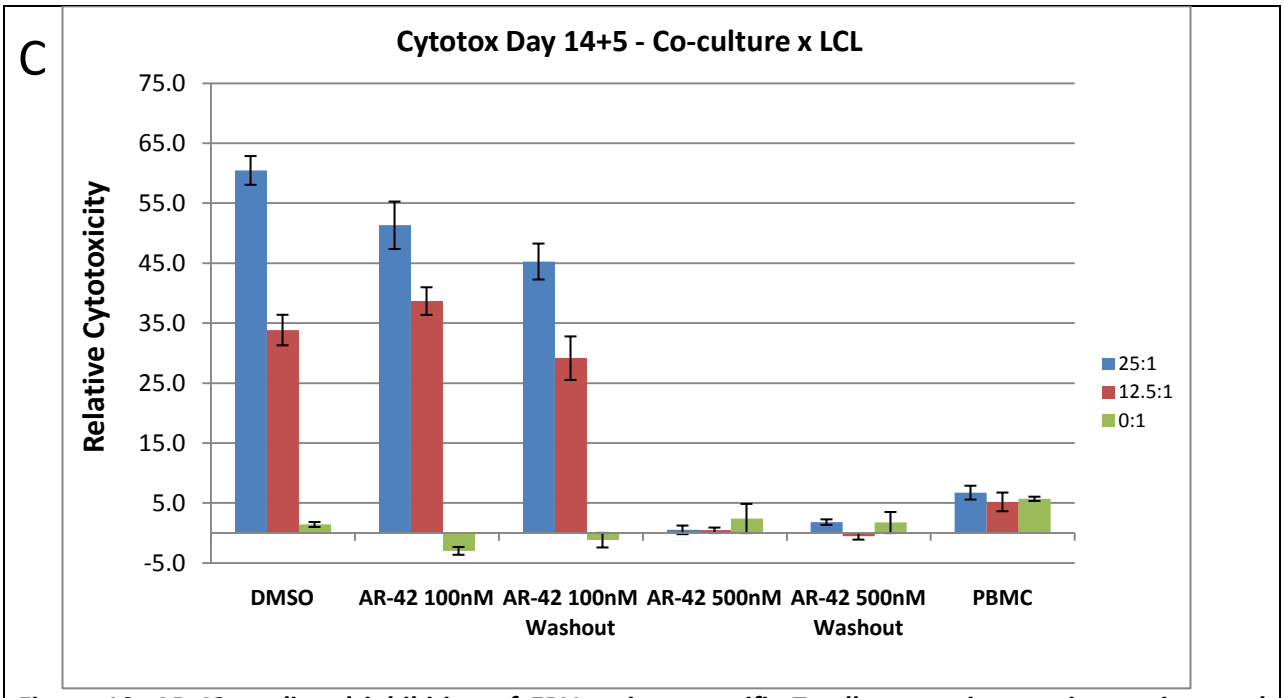
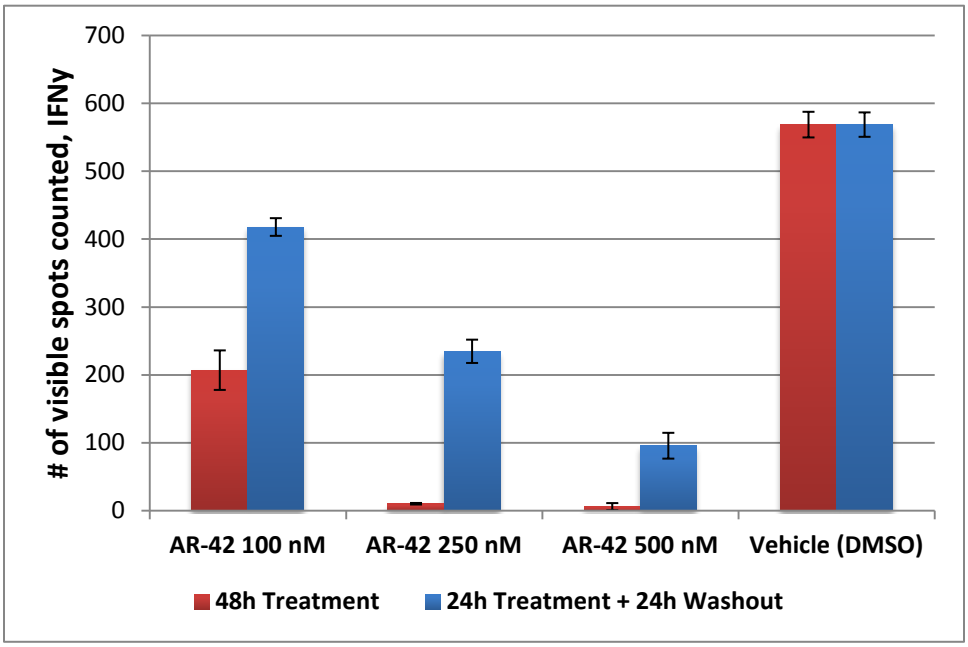


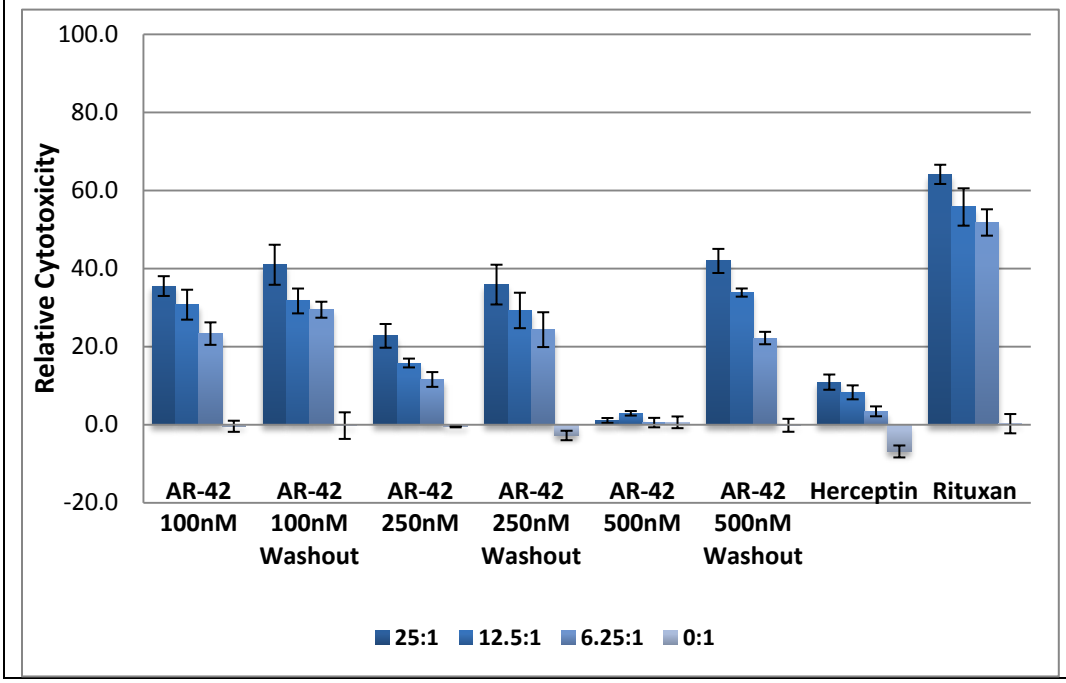
Figure 10. AR-42-mediated inhibition of EBV-antigen specific T cell responsiveness is transient and reversible.
(A) Activated EBV-specific T cells expanded in the presence of IL-2 and AR-42 were further re-stimulated with LCLs in the presence and absence of AR-42 for 5 days. T cells were stimulated in an ELISPOT assay with LCLs for 18 hours and checked for IFNγ and (B) granzyme B release. (C) CTLs with long-term 19 day treatment or 14 day treatment with a 5 day washout ran through a Cr-51 cytotoxicity assay.

To determine if AR-42 inhibitory effect was transient and/or reversible, we utilized expanded EBV-specific CTLs and checked for IFNγ and GzB production via ELISPOT. Activated EBV-specific T cells expanded in the presence of AR-42 were further incubated in media with and without AR-42 for 5 days. T cells were stimulated with EBV+ LCLs in an ELISPOT assay to determine IFNγ and GzB secretion potentials. Modest recovery of IFNγ and GzB production was observed in the washout samples at 100 nM AR-42 (Figure 10A,B). The data suggests long-term exposure to 500 nM AR-42 results in eventual loss of cytotoxic function of activated T cells. The long-term exposure inhibition/recovery CTL trend was also reflected in a corresponding four hour 51Cr release assay (Figure 10C).

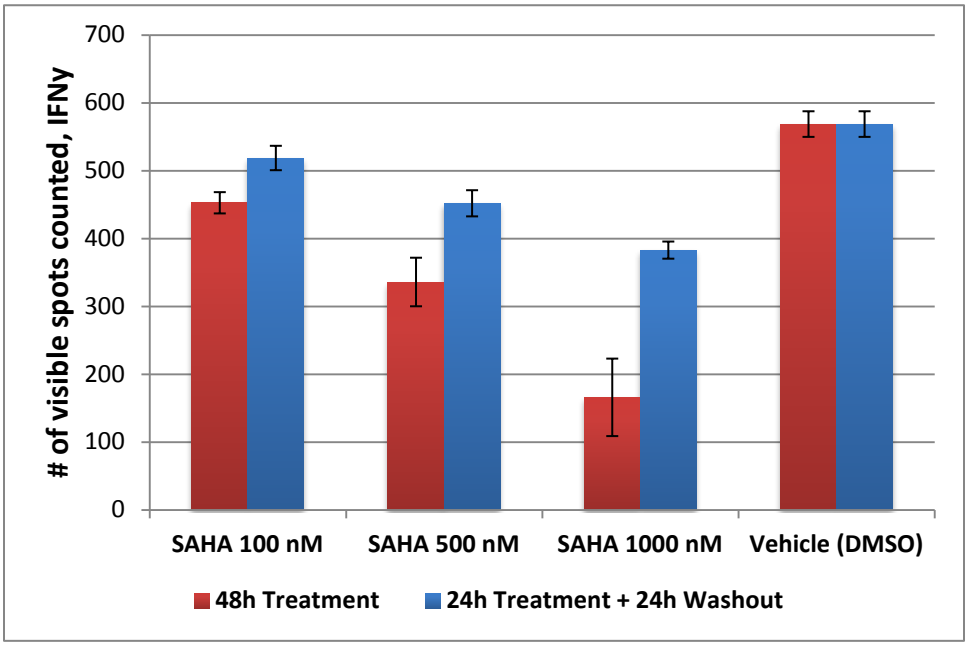
HDAC-I inhibition of NK cell cytokine production and cytotoxic function is transient and reversible

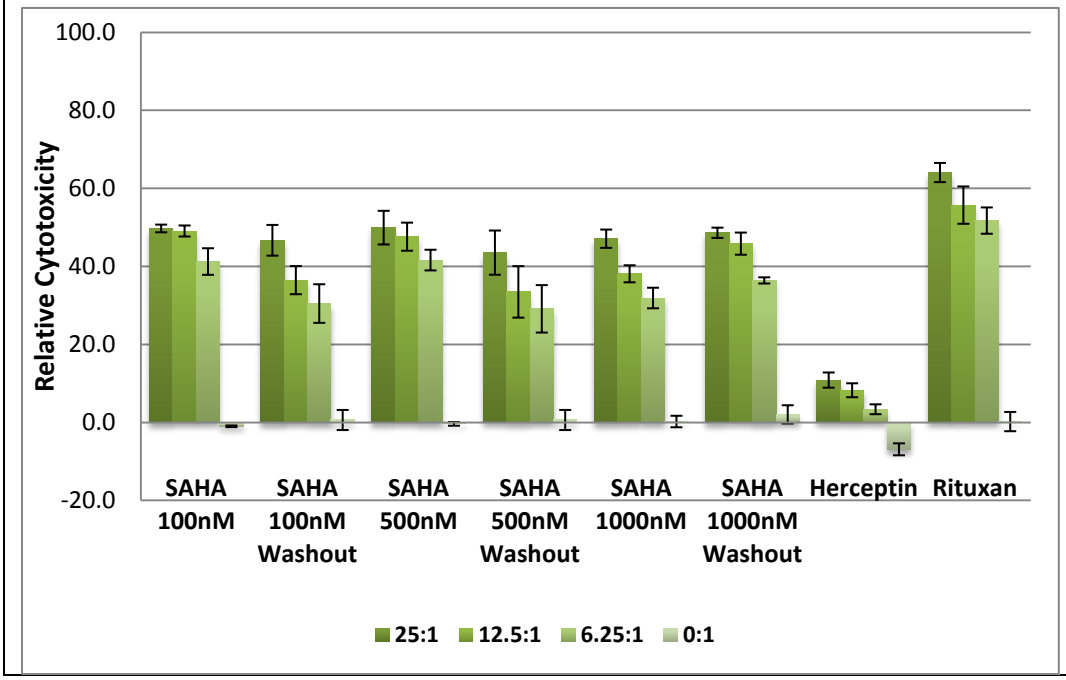
11A



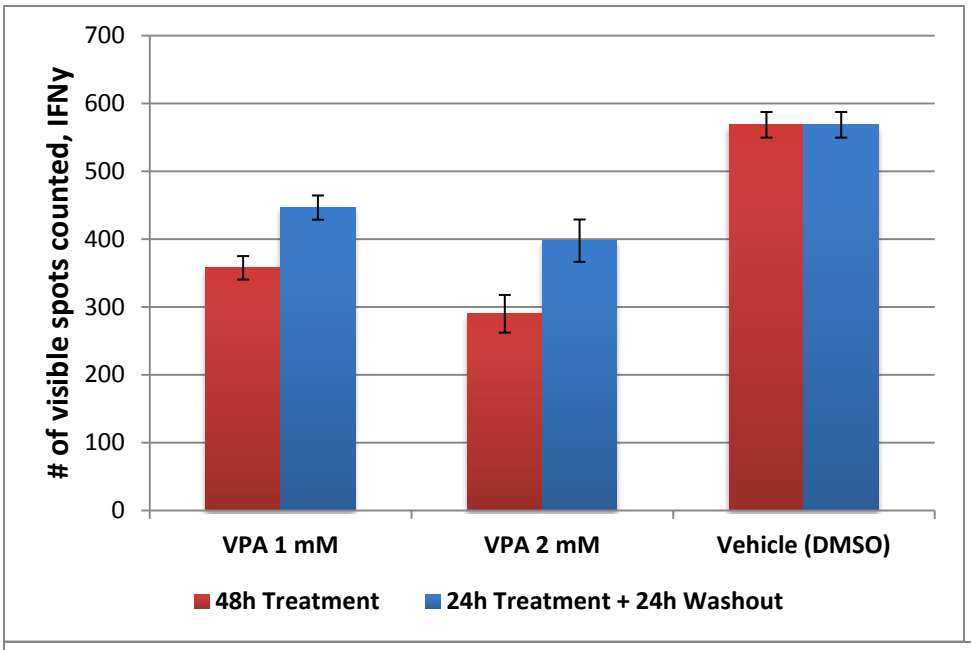


11B


700
600
500
400
300
200
100
0
# of visible spots counted, IFNy
SAHA 100 nM
SAHA 500 nM
SAHA 1000 nM
Vehicle (DMSO)
48h Treatment
24h Treatment + 24h Washout


100.0
80.0
60.0
40.0
20.0
0.0
-20.0
Relative Cytotoxicity
SAHA 100nM
SAHA 100nM Washout
SAHA 500nM
SAHA 500nM Washout
SAHA 1000nM
SAHA 1000nM Washout
Herceptin
Rituxan
25:1
12.5:1
6.25:1
0:1

11C



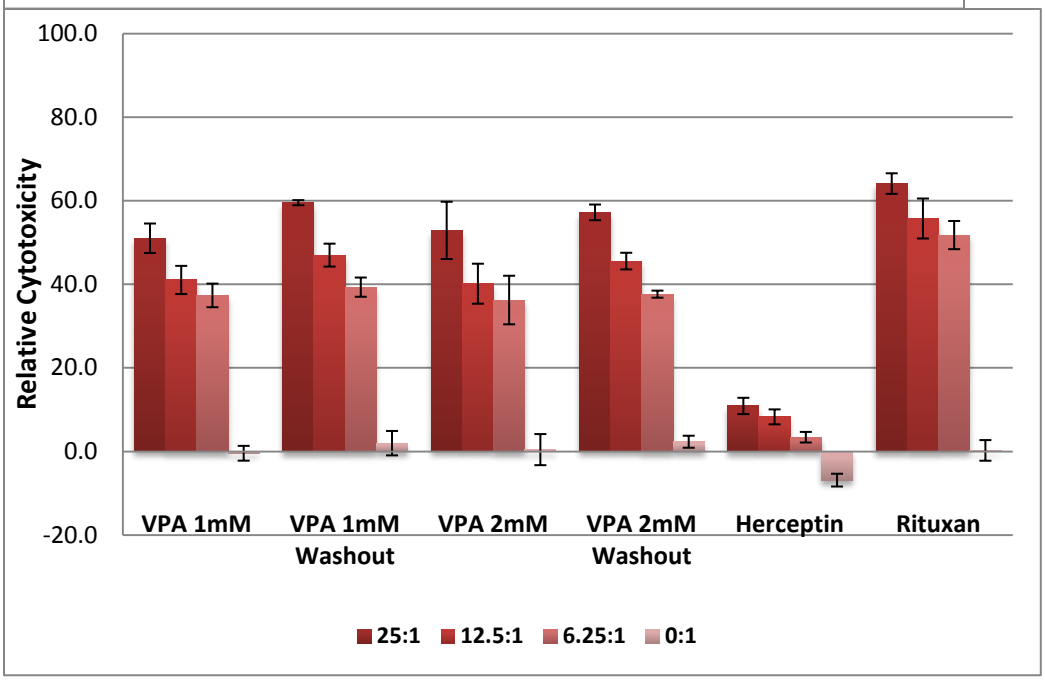


Figure 11. HDAC-I inhibition of NK cell cytokine production and cytotoxic function is transient and reversible.

(A) Purified NK cells treated with AR-42 and IL-2 for 48 hours or 24 hours with a 24 hour washout were examined in an IFNγ ELISPOT and ^{51}Cr release cytotoxicity assay. (B) SAHA. (C). Valproate.

To further investigate AR-42's washout potential, we checked NK cytotoxicity after short-term exposures with washouts with AR-42, SAHA, and valproate (Figure 11). Briefly, purified NK cells were treated with 100-500 nM AR-42, 100-1000nM SAHA, or 1-2mM valproate for either 48 hours continuously or through a 24 hour treatment + 24 hour washout regimen. Cells were analyzed in an IFNγ ELISPOT assay or a 51Cr release cytotoxicity assay.

Compared to the control (569 spots), the 100, 250, and 500 nM AR-42-treated NK cells resulted in fewer spots (207, 10, and 6 spots) indicative of substantially decreased IFNγ secretion from NK cells. The corresponding AR-42 washout conditions revealed significant recovery of NK cytotoxic function (418, 235, 96 spots, respectively). A similar inhibitory effect and functional recovery was present in the 100, 500, and 1000 nM SAHA-treated NK cells (452, 366, 166 spots). Valproate did not inhibit NK IFNγ secretion to the same degree as AR-42 or SAHA, despite its high concentrations of 1 and 2 mM (446, 238 spots).

The cytotoxic activity of 100, 250, and 500 nM AR-42–treated NK cells followed a dose-dependent trend of inhibition (36, 23, & 1%, respectively) as compared to the control at the max E:T ratio (64%). However, the corresponding AR-42 washout conditions recovered the NK cytotoxic activity to a near uniform level (41, 36, & 42%). These data suggest short-term exposure to AR-42 permits significant recovery of NK cytotoxic activity, albeit not to the same level as the untreated control. No significant ADCC inhibition was observed in the SAHA and valproate conditions.

Discussion

AR-42 exhibits a clear dose-dependent inhibitory effect on EBV-LCL growth at physiologically relevant nanomolar concentrations. Doses in the nanomolar concentrations exhibiting selective inhibitory effects are desirable and could indicate the ability to reduce or eradicate tumor viability without disturbing healthy host cell viability. Although AR-42's inhibitory effect on EBV-LCL growth holds significant promise for clinical applications, AR-42 also exerts a complete knockdown of NK and CTL cytotoxic functionality at 500 nM upon overnight incubation. This knockdown is observed consistently in Cr-51 chromium release assays, IFNy release assays, and granzyme B release assays at similar concentrations. Conversely, 500 nM AR-42 on EBV-LCLs only reduced viability to 75% after 48 h. Such a reduced cytotoxic potential for NK cells and CTLs could lead to detrimental patient effects as both NK and T cells are critical for maintaining healthy host functionality against biological pathogens from viral or bacterial infections.

Upon removal of AR-42 from the culture medium, NK and CTL cytotoxic functionality returned based on our assay results. These data suggest AR-42 inhibition of NK and CTL cytotoxic function and cytokine production is transient and reversible. While the extent of NK and CTL recovery appears to be dependent on the concentration and duration of AR-42 exposure, with long term AR-42 exposure to CTLs resulting in little recovery, a short-term 24 h exposure and 24 h washout to AR-42 appears to enable a significant portion of NK cytotoxic functionality to return.

It will be important to more clearly understand the immune modulatory activity of AR-42 for several reasons. First, our data suggest that there is great potential for designing an application to use HDACi drugs to achieve high anti tumor efficacy with minimal consequences on immunity. Secondly, there is potential for designing a reversible, quick-acting immunosuppressive agent to treat autoimmune disorders, where immune systems need to be inhibited for healthy host functionality. The second point is of particular interest as immunologists are continually searching for a potent and reversible immune-inhibiting agent which modulates a host cell's pre-existing cellular mechanisms [10]: Our work with AR-42 suggests that this may be possible fit this requirement.

We began our investigation of AR-42's immune-inhibitory mechanism by examining the expression of common EBV-LCL, NK, and T cell activating and deactivating regulatory receptors. AR-42 resulted in little to no changes in the programmed death receptor-ligand pathway between T cells and EBV-LCLs, or the MHC I expression on EBV-LCLs. AR-42 slightly downmodulated expression of CD16 (Fc antibody binding), KIR (NK deactivating), and NKG2D (NK activating) receptors. AR-42 resulted in no changes in NKp30 or NKp46 (NK activating) expression. Given the lack of any clear observable patterns and conflicting results indicating AR-42 mediated-downmodulation of two key activating and deactivating NK receptors (NKG2D, KIR), the data suggests regulatory NK and CTL receptors are not the primary mechanism behind AR-42 immunosuppressive effects. In addition, a regulatory receptor mechanism would be difficult to describe the rapid knockdown of effector cytotoxic functionality occurring within overnight incubation periods at low doses of AR-42.

However, the use of several HDAC-I drugs in an NK washout experiments testing for Cr-51 cytotoxic potential and IFNy release holds considerable promise for unlocking AR-42's immunosuppressive mechanism. As stated previously, AR-42, SAHA, and valproate are all Zn2+ based short-chain fatty acid HDAC inhibitors. AR-42 and SAHA exhibit both class I HDAC (HDAC1, 2, 3, 8) and class II HDAC (HDAC4, 5, 6, 7, 9, 10) specificity while valproate exhibits strictly class I HDAC specificity. [18, 19] Differential effects are observed in NKs treated with each HDAC inhibitor: AR-42 inhibits ADCC and IFNy release; SAHA inhibits IFNy release; valproate appears to slightly inhibit IFNy at a much larger millimolar concentration. These data suggests class I HDAC enzyme inhibition alone does not play a significant role in modulating the innate and adaptive immune cytotoxic response. However, the ability of AR-42 to exert a strong inhibitory effect on ADCC, unlike SAHA, indicates AR-42's immune-suppressing mechanism may be causing deacetylation of non-histone proteins as described in current literature. [10]

Future Directions

The preliminary data suggests AR-42 exerts similar inhibitory effects on both the innate and adaptive immunity. However, little is known of HDAC inhibition on other cells within the immune system including antigen presentation or cytokine/chemokine secretion. Further study into the effects of HDAC inhibition upon T cell function and migration would serve as a possible starting point.

The differences in HDAC enzyme class-specific inhibition indicates each individual HDAC enzyme may hold unique immunosuppressive and anti-tumor activity. Unlocking the specific mechanism behind AR-42's reversible inhibitory effect could result in a potent therapeutic for autoimmune disorders. The observed differential results of broad (AR-42, SAHA), and narrow (valproate) spectrum HDAC inhibitors on host immune function encourages further investigation. An examination into the acetylation status of various histones affected by individual HDAC enzymes may help to reveal the mechanism functioning at the genomic level.

The data suggests that clinical trials utilizing AR-42 or other HDAC inhibitors should exercise caution when treating patients to prevent non-desirable immune-suppressive activity. Future experiments using samples taken from patients treated with HDAC inhibitors will increase our understanding of how HDAC effects immune function and tumor suppression. This data would lead to the development of delivery and dosing schemas to maximize anti tumor activity while

minimizing immune suppression. We would also be able to modify such schemes dependent on the clinical scenario, which is very important when delicately balanced treatments are essential. For example, in post-transplant lymphoproliferative disorder (PTLD), increased immune suppression to prevent organ rejection comes at the price of reduced anti-tumor activity. Clinicians need to be mindful of both.

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